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BRIEF REPORT

Functional expression of the recombinant ATPase of orf virus

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Abstract Nucleotide sequence analysis has indicated that the A32L gene of orf virus can encode an ATPase (Chan et al. in Gene 432:44–53, 2009). In this work, we cloned the A32L gene into a prokaryotic expression vector, and the recombinant protein was expressed and purified. The antigenicity of recombinant ATPase was examined by immunoblotting, and its identity was confirmed by mass spectrometry. The ATP hydrolysis function of the purified recombinant protein was examined, and our results showed that it exhibited the ATPase activity. Similar to other viral ATPases, the ATPase of orf virus remained active in the presence of different divalent ions; nevertheless, unlike other viral ATPases, our recombinant ATPase exhibited similar enzymatic activity in reaction buffers of different pH.

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Orf virus is classified as a member of the genus *Parapoxvirus* and has a linear double-stranded DNA genome of approximately 134–139 kbp. The orf virion is about 260 nm in length and 160 nm in width. The virion shape is ovoid with a crisscross pattern on its surface. Viral DNA replication, transcription, and virion assembly occur in the cytoplasm [5, 6, 9, 14, 16].

Orf virus infection can give rise to contagious ecthyma in goats, sheep, and other ruminants. The clinical signs of the disease are proliferative lesions around the muzzle and lips; occasionally the gums and tongue are affected, which is frequently found in young lambs. The disease lasts about 3–4 weeks, and affected animals may recover in 1–2 months. The mortality rate is approximately 10% in lambs and 93% in baby goats [8, 9, 13, 15, 18].

The A32L gene (following the nomenclature of vaccinia virus), open reading frame 108, is near the right end of the map of the orf virus genome. The A32L gene is predicted to encode an ATPase that may be involved in packaging of viral DNA into virions [2, 10, 11]. In vaccinia virus, the viral ATPase is a core-associated enzyme [16]. An A32 deletion mutant of vaccinia virus has been shown to reduce viral DNA packaging [2]. Although the A32L nucleotide sequence of orf virus is similar to that of the A32 gene of vaccinia virus [4], the role of the A32L gene in orf viral DNA packaging has not been studied.

Based on our previous sequence analysis of the A32L gene [4], we have further investigated the biochemical function of the recombinant A32L gene in this study. Cloning, expression, purification and identification of the A32L gene product were carried out, and the biochemical results demonstrated that the recombinant protein expressed from the cloned A32L gene displayed ATPase activity. To our knowledge, this work is the first report of biochemical analysis of orf virus ATPase.

Total DNA was extracted from skin lesions from a goat infected with orf virus, and subsequently, the targeted A32L gene of the Taiping strain (GenBank accession number EU327510) was cloned into the pCRII vector (Invitrogen) as described previously [3, 4]. For further cloning of the A32L gene into an expression vector, the A32L gene was amplified by PCR with one set of primers (F primer: GGAGATATGGATCCGGATGTCGTGCAG; R primer: GGCGCTCAGCGGCCGCATTGCCGCCG) that were designed to incorporate *Bam*HI and *Not*I restriction enzyme sites. PCR was conducted following the manufacturer's instructions (TOYOBO; Blend-Taq) at a final concentration of 0.2 mM dNTP, 0.2 μM of each primer, 2 mM Tris–HCl, 10 mM KCl, and 2 mM $MgCl_2$. The PCR program was started with an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 59°C for 1 min, and an extension at 72°C for 45 s, and it was ended with a final extension at 72°C for 7 min.

For construction of the expression plasmid, both the PCR product and the pET32b vector (Novagen) were digested with *Bam*HI and *Not*I restriction enzymes at 37°C for 2 h. The subsequent ligation between pET32b and the insert containing the A32L gene was done using T4 DNA ligase, and the resulting plasmid is illustrated in Fig. 1a. Competent cells were then transformed with the ligation mix and spread onto an ampicillin-selection plate. Six colonies were selected and grown in 4 mL ampicillin-containing broth at 37°C for 6 h. The identity of plasmids was examined by *Bam*HI and *Not*I digestion and automated DNA sequencing.

For protein expression, BL21 AI bacteria (Invitrogen) were transformed with the constructed plasmid, and expression was induced by the addition of isopropyl *β*-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.8 mM and 0.2% L-arabinose (CALBIOCHEM) at 16°C for 24 h. Then, the bacterial pellet was collected, resuspended in lysis buffer (0.05 M Tris–HCl, 0.5 M NaCl, 10 mM imidazole, pH 7.4), subjected to three cycles of freezing and thawing, and sonicated on ice for 6 min. The supernatant containing the native form of the recombinant protein was recovered by centrifugation for 30 min at 4°C and then purified using the chelating Sepharose Fast Flow (GE Healthcare) according to the manufacturer's instructions with modifications. In brief, the crude bacteria lysate was mixed with Ni^{2+}-charged Sepharose and rocked at 4°C for 2 h. The matrix was washed several times with 10 ml wash buffer (0.05 M Tris–HCl, 0.5 M NaCl, 20 mM imidazole, pH 7.4) to remove non-specifically bound endogenous histidine-containing proteins, and finally, the desired recombinant His-tag A32L protein was eluted in 4 mL elution buffer (0.05 M Tris–HCl, 0.5 M NaCl, 400 mM imidazole, pH 7.4).

The purified protein was examined by western blotting. The primary antibody was mouse anti-His·Tag antibody (AbD Serotec), and the secondary antibody was a goat anti-mouse antibody conjugated with horseradish peroxidase (HRP). Detection was performed following the manufacturer's instructions (ECL Chemiluminescence System, Amersham, GE Healthcare) and visualized using X-ray films. In addition, the ATPase identity of the recombinant A32L protein was verified by mass spectrometry analysis (Mission Biotech, Taipei, Taiwan).

The purified recombinant protein was dialyzed in PBS to remove urea and imidazole. The concentration of the recombinant protein was determined by the Bradford method (Bio-Rad). For functional assays, the protein (1, 2 or 4 μg) was mixed with 10 μl of 3× assay buffer

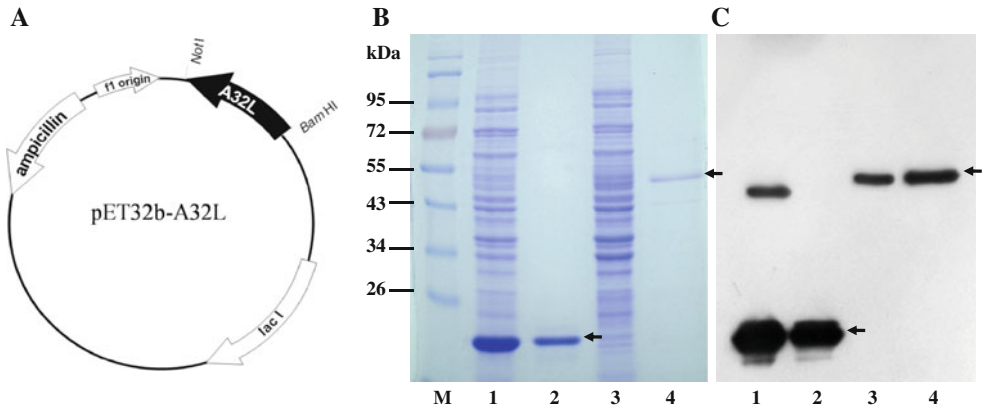

Fig. 1 Map of the A32L gene inserted into the pET32b plasmid for protein expression (**a**). Purification and immunoblot of the recombinant A32L protein (**b**, **c**). The lysate and the purified protein were separated by SDS-PAGE and stained with Coomassie blue (**b**). Western blot with mouse antibody against-His-tag (**c**). *Lane 1* thioredoxin expressed from the pET32b vector as a positive control, *lane 2* purified thioredoxin; *lane 3* recombinant A32L protein expressed in crude bacterial lysate, *lane 4* purified recombinant A32L protein, *M* prestained protein markers (Bio-Rad). The 50 kDa A32L protein and thioredoxin (20 kDa) are indicated by *arrows*

(150 mM HEPES, pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$) and 0.15 μCi of radioactive [α-^{32}P]ATP in a total final volume of 30 μl and incubated at 37°C for 30 or 60 min [20]. Then, 5 μl of each reaction mixture was spotted onto a polyethyleneimine (PEI) cellulose thin-layer chromatography (TLC) plate (Merck) and developed in a solvent system (1 M acetic acid and 5 M LiCl). Finally, an autoradiogram was obtained by exposing the TLC plate to an X-ray film.

To express the recombinant ATPase protein, host cells were transformed with plasmid pET32b-A32L, and expression was induced by the addition of IPTG and L-arabinose. The A32L recombinant protein was expressed and purified (Fig. 1b), and the recombinant protein displayed a molecular size of approximately 50 kDa on 10% SDS-PAGE, as expected. Using a mouse anti-His-tag antibody, the recombinant protein was examined by western blotting, which demonstrated that the recombinant

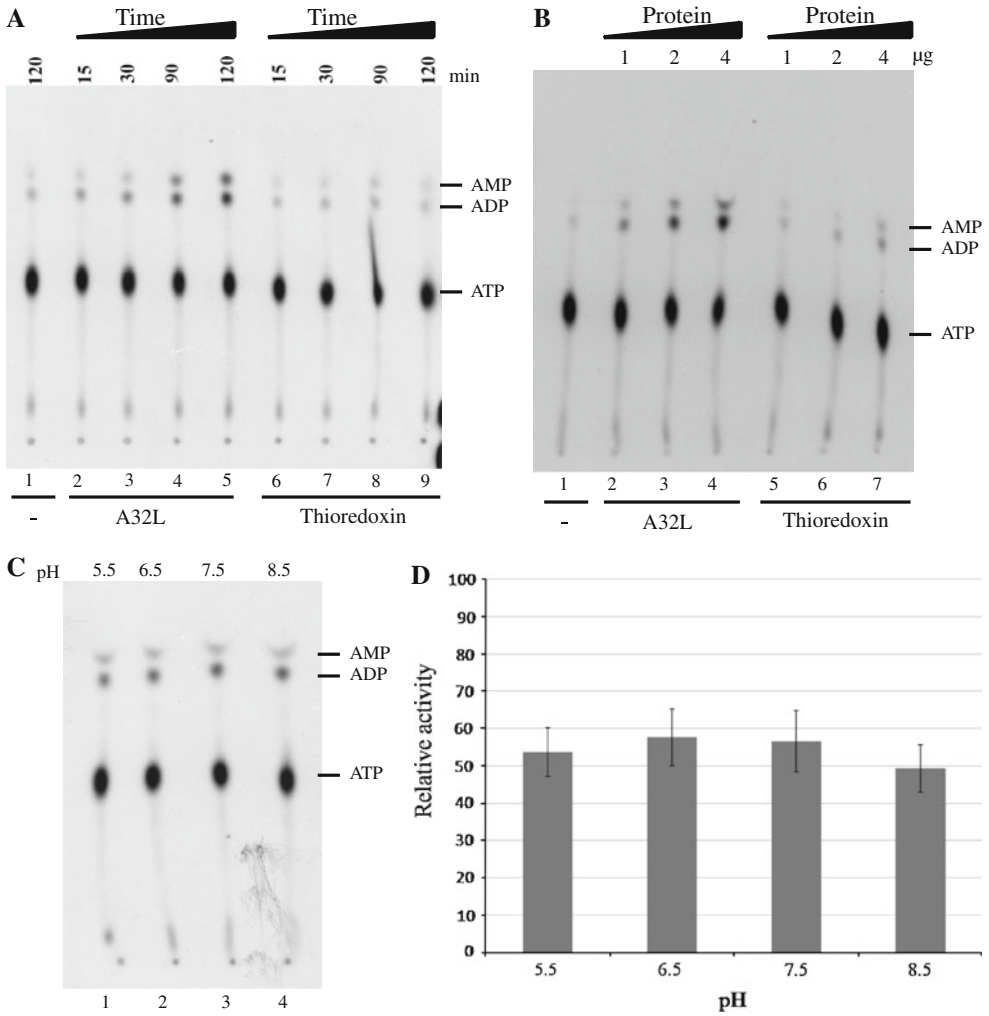

Fig. 2 Functional assay of recombinant A32L protein showing its ATPase activity. **a** Each reaction contained 1 μg of recombinant protein and 0.15 μCi of [α-^{32}P]ATP. From *lane 2–5*, increasing ATPase activity was observed with increasing reaction time, as indicated by the appearance of ADP and AMP products. Compared with the recombinant ATPase, the tag protein, thioredoxin, (*lane 6–9*), as the background control, did not show ATPase activity. *Lane 1* reaction mixture containing [α-^{32}P]ATP only and no additional proteins (negative control). **b** The effect of enzyme concentration on ATPase activity. These reactions were carried out with different protein concentrations (1, 2, and 4 μg), and the reaction mixtures were incubated at 37°C for 30 min. The A32L protein demonstrates increasing ATPase activity that is dose-dependent (*lanes 2–4*). The thioredoxin expressed from the empty pET32b vector (*lanes 5–7*) served as a background control. *Lane 1* reaction buffer including only [α-^{32}P]ATP as a negative control. **c** ATPase activity of recombinant protein in buffers with different pH values. Reactions were conducted in assay buffers of different pH values (5.5, 6.5, 7.5 and 8.5); 2 μg of recombinant protein was used for each reaction, and the reaction mixtures were incubated at 37°C for 30 min. The results demonstrated similar ATPase activities of recombinant protein at different pH values. **d** Quantitation of ATPase activity analyzed using the FLUORCHEM SP computer program. The values are the means of three independent reactions, and *error bars* are standard errors of the means

protein could react with the antibody (Fig. 1c). The identity of the purified recombinant protein was confirmed by mass spectrometry, and partial amino acid sequence data indicated that our recombinant protein was an ATPase (data not shown).

To determine whether the purified recombinant A32L protein possessed ATPase activity, the ATPase function of our recombinant protein was assayed by its ability to hydrolyze ATP. The reaction products were analyzed by TLC. As shown in Fig. 2a, ATPase activity could be observed, with hydrolysis of ATP and production of ADP and AMP, and this enzymatic activity increased in proportion to the incubation time. In addition, A32L also showed more ATP hydrolysis with increasing concentration of the recombinant protein (Fig. 2b). In contrast, the tag protein thioredoxin produced from the empty vector pET32b did not display ATPase activity when the incubation time or protein concentration in the reaction were increased (Fig. 2a, b).

From nucleotide sequences analysis, it is known that the A32L gene of orf virus is homologous to the A32 gene of vaccinia virus [4, 11]. However, the in vitro ATPase function of vaccinia virus A32 gene has not been reported yet. Our results provide experimental evidence that the A32L gene of orf virus encodes a functional ATPase in vitro.

Next, the effect of different pH conditions on enzyme activity was examined. The results demonstrated that the recombinant ATPase could hydrolyze ATP in buffer of pH 5.5, 6.5, 7.5 or 8.5 with almost the same activity (Fig. 2c). It is unusual that an enzyme retains similar activity over such a wide pH range. In contrast, studies of several other viral ATPases have shown that their enzyme activity varies significantly as the pH changes [12, 17, 20].

The effect of metal ions on the ATPase activity of our protein was also studied. It was found that the recombinant ATPase had similar enzymatic activity in the presence of Mg, Ca or Mn ions in the reaction buffer (Supplementary information).

The nucleotide sequences of the A32L gene of orf virus (Taiping strain; GenBank accession number EU327510) and of the A32 of vaccinia virus (Copenhagen strain; GenBank accession number M35027.1) share about 82% similarity [4], and the deduced amino acid sequences contain highly conserved functional motifs of ATPases, suggesting that they have similar biochemical properties and a similar biological function in virus DNA packaging. Deletion analysis of the vaccinia virus A32 gene resulted in noninfectious, DNA-deficient viral particles [2]. However, the roles of the A32L gene product in DNA packaging and morphogenesis of the orf virion remain unknown.

Vaccinia virus can produce at least two other ATPases that are DNA-dependent. One is encoded by the A18R gene and has DNA helicase activity [1, 19], and the other, called NPH-I (nucleoside triphosphate phosphohydrolase-I), contains a DExH box and plays a role in termination of transcription [7]. The homologues of A18R-encoded ATPase and NPH-1 ATPase of vaccinia virus in orf virus have not been studied yet.

In this work, we successfully cloned and expressed the A32L gene into a pET32b expression vector, and its ATPase activity was demonstrated. Interestingly, under different pH conditions, our recombinant ATPase displayed similar levels of activity. This finding differs from what has been observed with of other viral ATPases [12, 17, 20].

References

1. Bayliss CD, Condit RC (1995) The vaccinia virus A18R gene product is a DNA-dependent ATPase. J Biol Chem 270:1550–1556
2. Cassetti MC, Merchlinsky M, Wolffe EJ, Weisberg AS, Moss B (1998) DNA packaging mutant: repression of the vaccinia virus A32 gene results in noninfectious, DNA-deficient, spherical, enveloped particles. J Virol 72:5769–5780
3. Chan KW, Hsu WL, Wang CY, Yang CH, Lin FY, Chulakasian S, Wong ML (2009) Differential diagnosis of orf viruses by a single-step PCR. J Virol Methods 160:85–89
4. Chan KW, Yang CH, Lin JW, Wang HC, Lin FY, Kuo ST, Wong ML, Hsu WL (2009) Phylogenetic analysis of parapoxviruses and the C-terminal heterogeneity of viral ATPase proteins. Gene 432:44–53
5. Damon IK (2007) Poxviruses. In: Knipe DM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE (eds) Fields virology. Lippincott, Williams and Wilkins, Philadelphia, pp 2947–2975
6. Delhon G, Tulman ER, Afonso CL, Lu Z, de la Concha-Bermejillo A, Lehmkuhl HD, Piccone ME, Kutish GF, Rock DL (2004) Genomes of the parapoxviruses ORF virus and bovine papular stomatitis virus. J Virol 78:168–177
7. Deng L, Shuman S (1998) Vaccinia NPH-I, a DExH-box ATPase, is the energy coupling factor for mRNA transcription termination. Genes Dev 12:538–546
8. Gumbrell RC, McGregor DA (1997) Outbreak of severe fatal orf in lambs. Vet Rec 141:150–151
9. Haig DM, Mercer AA (1998) Ovine diseases. Orf Vet Res 29:311–326
10. Iyer LM, Aravind L, Koonin EV (2001) Common origin of four diverse families of large eukaryotic DNA viruses. J Virol 75:11720–11734
11. Koonin EV, Senkevich TG, Chernos VI (1993) Gene A32 product of vaccinia virus may be an ATPase involved in viral DNA packaging as indicated by sequence comparisons with other putative viral ATPases. Virus Genes 7:89–94
12. Kuo MD, Chin C, Hsu SL, Shiao JY, Wang TM, Lin JH (1996) Characterization of the NTPase activity of Japanese encephalitis virus NS3 protein. J Gen Virol 77(Pt 9):2077–2084
13. Mazur C, Machado RD (1989) Detection of contagious pustular dermatitis virus of goats in a severe outbreak. Vet Rec 125:419–420
14. Mercer AA, Ueda N, Friederichs SM, Hofmann K, Fraser KM, Bateman T, Fleming SB (2006) Comparative analysis of genome sequences of three isolates of Orf virus reveals unexpected sequence variation. Virus Res 116:146–158

15. Mondal B, Bera AK, Hosamani M, Tembhurne PA, Bandyopadhyay SK (2006) Detection of Orf virus from an outbreak in goats and its genetic relation with other parapoxviruses. Vet Res Commun 30:531–539
16. Moss B (2007) Poxviridae: the viruses and their replication. In: Knipe DM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE (eds) Fields virology. Lippincott, Williams and Wilkins, Philadelphia, pp 2905–2945
17. Ramadevi N, Roy P (1998) Bluetongue virus core protein VP4 has nucleoside triphosphate phosphohydrolase activity. J Gen Virol 79(Pt 10):2475–2480
18. Reid HW (1991) Disease of sheep, 2nd edn. Oxford, Blackwell scientific publication, London
19. Simpson DA, Condit RC (1995) Vaccinia virus gene A18R encodes an essential DNA helicase. J Virol 69:6131–6139
20. Su YP, Shien JH, Liu HJ, Yin HS, Lee LH (2007) Avian reovirus core protein muA expressed in *Escherichia coli* possesses both NTPase and RTPase activities. J Gen Virol 88:1797–1805